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Fifteenth day of July 2004

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PROVISIONAL SPECIFICATION

Invention Title:

Method For Detection Of Alkylated C*ytosine In DNA*

The invention is described in the following statement:

Method For Detection Of Alkylated Cytosine In DNA

Field of the Invention

The present invention relates to methods for detecting alkylated cytosine in DNA. Methods of the invention employ enzymes that differentially modify alkylated cytosine and cytosine. The presence of alkylated cytosine in DNA is determined by evaluating the level of enzymatic modification of the DNA following incubation of the DNA with at least one such enzyme. The detection of alkylated cytosine in DNA is useful for diagnostic and other purposes.

Background of the Invention

At least seven different covalent base modifications have been identified in prokaryotic, eukaryotic, bacteriophage and/or viral genomes (1). In higher order eukaryotes the most abundant covalently modified base is 5-methylcytosine located 5' to guanosine in CpG dinucleotides. It has been hypothesised that methylation patterns play a role in gene transcription, X chromosome inactivation, genomic imprinting, cell differentiation and tumourigenesis (2).

The abnormal phenotype of cancer cells is due to both qualitative and/or quantitative change. Sequence-based qualitative changes (genetic mutations) are preserved in the genomic DNA and this has facilitated their detection and characterisation. The inheritance of information on the basis of gene expression is known as epi-genetics. Methylation of cytosine bases in nucleic acid can effect epigenetic inheritance by altering expression of genes and by transmission of DNA methylation patterns through cell division. Cancer cells have been frequently shown to harbour both genetic and epi-genetic mutations.

Neoplastic cells simultaneously harbour multiple abnormalities relating to methylation patterns. They frequently have both widespread genomic hypomethylation as well as more regional areas of hypermethylation (1). Regional methylation of normally unmethylated CpG islands located within promoter regions of genes has been shown to be correlated with the down regulation of the corresponding gene. This hypermethylation can serve as an alternative mechanism to coding region mutations for the inactivation of

tumour suppressor genes. Examples of genes which have CpG island hypermethylation in association with human tumours include p16 (lung, breast, colon, prostate, renal, liver, bladder, and head and neck tumours), *E-cadherin* (breast, prostate, colon, bladder, liver tumours), the von Hippel Lindau (VHL) gene (renal cell tumours), *BRCA1* (breast tumours), p15 (leukemias, Burkitt lymphomas), hMLH1 (colon), ER (breast, colon, lung tumours; leukemias), HIC1 (brain, breast, colon, renal tumours), MDG1 (breast tumours), GST-π (prostate tumours), O^6-MGMT (brain tumours), calcitonin (carcinoma, leukemia),and *myo-D* (bladder tumours) (1, 3).

The converse situation has also been reported, whereby CpG hypomethylation is thought to contribute to neoplastic progression. The urokinase CpG island was found to be hypermethylated in early stage, non-metastatic breast tumour cells but was hypomethylated in highly metastatic breast tumor cells (4). Similarly, hypomethylation of a region within the metastasis-associated S100A4 gene has been hypothesized as the mechanism of gene activation in colon adenocarcinoma cell lines (5).

At least eight different methods, along with several variations, allow characterisation of 5-methylcytosine or related modified bases in DNA genomes (2). Each method has advantages and disadvantages in terms of specificity, resolution, sensitivity and potential artefacts.

The total nucleic acid base composition of a genome can be determined by hydrolysing DNA to its constituent nucleotides, either chemically or enzymatically, and then fractionating and analysing the composition by standard methods (chromatography, electrophoresis and high pressure liquid chromatography). This approach quantifies the amount of modified bases present in the genome, but does not give any information on which part of the genome was originally modified. Dinucleotide composition and frequency can be determined by nearest-neighbour analysis, but again this method produces only limited sequence information. Neither of these methods are genome specific, and contamination of samples by DNA from viruses and other endoparasites can lead to misleading results.

More specific methods exist which can provide data on exactly where in the sequence of the genome modified bases exist. Genomic DNA can be analysed by restriction enzymes that are sensitive to methylation. With this method, however, the

number of sites that can be examined is limited by the number of sequences recognized by methylation sensitive restriction enzymes. Sequencing would provide sequence-specific information, but methylation patterns are not preserved during PCR or when eukaryotic DNA is amplified in bacteria through molecular cloning.

It is necessary to differentially modify the bases, in a methylation specific manner, to produce a modified sequence where the methylation-specific changes are retained during sequencing protocols. There are currently three protocols that rely on analysis of differential base modification. All of these protocols involve modification of DNA, induced by chemical treatment of the DNA followed by analysis of the DNA sequence. Hydrazine (N_2H_4), permanganate (MnO_4^-), and bisulfite (HSO_3^-) all differentially modify cytosine bases in genomic DNA depending on the methylation status of the cytosine base.

Hydrazine has a lower reactivity with 5-methylcytosine than with cytosine or thymine. After incubation of DNA with hydrazine the DNA is run on a sequencing gel. Comparison of the hydrazine-treated DNA with DNA treated with other base-specific chemical cleavage compounds allows the sequence of the DNA to be determined. In hydrazine-treated DNA samples 5-methylcytosine-containing sequence positions produce an absence or reduced intensity of bands compared to the cytosine and cytosine + thymidine specific ladders of sequencing reactions from genomic DNA. Thus the hydrazine protocol produces a negative result that correlates with the presence of 5-methylcytosine. Unambiguous identification of 5-methylcytosine requires the generation of a positive signal. A further disadvantage of hydrazine modification for the identification of 5-methylcytosine is that μg of template DNA is required.

Potassium permanganate, at weakly acidic pH and room temperature, reacts preferentially with thymine and 5-methylcytosine, and only weakly with cytosine and guanine. After incubation of DNA with permanganate the DNA is run on a sequencing gel. Comparison of the permanganate-treated DNA with DNA treated with other base-specific chemical cleavage compounds allows the sequence of the DNA to be determined. Permanganate oxidation of DNA can therefore be used to discriminate between cytosine and 5-methylcytosine (6). Although the permanganate protocol produces a positive result, and thus has an advantage over the hydrazine protocol, permanganate does react weakly with cytosine and hence discrimination of cytosine versus 5-methylcytosine depends on a

difference in the intensities of their bands on the sequencing gel. A further disadvantage of permanganate modification is that μg of template DNA is again required.

Bisulfite treatment of genomic DNA deaminates unmethylated cytosine bases in the nucleic acid template to uracil, whereas 5-methylcytosine is resistant to deamination. Bisulfite has little activity on cytosine bases in double stranded DNA and so genomic double stranded DNA is preferably denatured to single stranded DNA. The standard bisulfite modification protocol uses incubation in alkali (NaOH) to denature double stranded DNA to single stranded DNA (7). Bisulfite deaminates cytosine slowly and incubation times have to achieve a compromise between complete deamination of all cytosine and fragmentation of DNA after long incubations. Protocols for bisulfite modification use a range of incubation times, generally from 4 to 20 hours incubation in bisulfite.

Grunau *et al* (8) studied optimum conditions for bisulfite-mediated deamination of cytosine and found that 4 hours incubation at 55°C gave 99% deamination of cytosine, but under these conditions 84 to 96% of the DNA was degraded, reducing yields for subsequent steps. Further, 5-methylcytosine is deaminated by heat at a greater rate than cytosine. For example, the rate of deamination of 5-methylcytosine at 60°C is 1.5 times higher than that of cytosine (9). Incubations in bisulfite at lower temperatures reduce fragmentation of DNA but the incubation times have to be extended to 14 to 20 hours to achieve full deamination of cytosines. Bisulfite modification requires approximately 10 ng of DNA for subsequent analysis using PCR-based methods.

The modified DNA sense and anti-sense strands produced by bisulfite modification are no longer complementary and therefore subsequent amplification by PCR must be performed with primers that are designed to be strand specific that is, the primers are complementary to either the modified sense strand or the modified anti-sense strand. When the region of interest is amplified by PCR, uracil (previously cytosine) is converted to thymine and 5-methylcytosine is converted to cytosine (7). The PCR products (amplicons) can be subsequently analysed by standard DNA sequencing (7) or other PCR-based techniques that produce sequence information such as methylation-specific PCR (10) or REMS-PCR (36), and analysis with restriction enzymes (3) or methylation-specific probes (11).

Although the bisulfite method has advantages in terms of ease of use and sensitivity over other existing protocols, potential artefacts can arise from the experimental protocol (2) namely not all cytosines are converted to uracil, a small percentage of 5-methylcytosine is converted to thymidine (12) (DNA polymerases do not distinguish between uracil and thymine) and there can be a loss of DNA from fragmentation caused by the long incubations and non-physiological buffers required (8). The full protocol is long and laborious involving 2 to 3 days of manipulation and at least 4 to 20 hours of incubation in bisulfite before results are obtained.

The rate-limiting step in all epigenetic studies is sample preparation using the bisulfite modification protocol.

DNA extracted from many types of specimens including normal and tumour tissue, paraffin embedded tissues, as well as plasma and serum has been shown to contain aberrantly methylated sequences using the combination of bisulfite treatment and analysis by PCR-based methods (4, 13, 14).

A variety of enzymes with the ability to deaminate cytosine bases have been described. Cytidine Deaminase (EC 3.5.4.5.) converts cytidine to uridine and is widely distributed in prokaryotes and eukaryotes. Cytosine Deaminase (EC 3.5.4.1.) converts cytosine to uracil. Deoxycytidine Deaminase (EC 3.5.4.14.) converts deoxycytidine to deoxyuridine and Deoxycytidilate Deaminase converts deoxycytidine-5-phosphate to deoxyuridine-5-phosphate. These enzymes show different degrees of substrate specificity depending on the source of the enzyme. The ability of Cytidine Deaminase and Cytosine Deaminase to discriminate between 5-methylcytidine and 5-methylcytosine and their unmethylated analogues as substrates (respectively) is species specific. Cytidine Deaminase from humans can deaminate, with varying efficiency, numerous cytidine derivatives including cytosine, deoxycytidine, and 5-methylcytidine (15, 16). Cytosine Deaminase from *Pseudomonas* can utilise 5-methylcytosine (17) while the enzyme produced by enterics can only use cytosine as a substrate. Cytosine Deaminase from the fungus *Aspergillus fumigatus* and the yeast enzyme can utilise 5-methylcytosine as a substrate (18, 19). The substrate specificity of these enzymes in lower eukaryotes such as *Drosophila* and nematodes, which do not contain 5-methylcytidine in their genome, is unknown.

Apolipoprotein B mRNA Editing Enzyme (ApoBRe) is the central component of an RNA editsome whose physiological role is specifically to deaminate the cytosine base at position #6666 of the *apoB* mRNA to uracil in gastrointestinal tissues creating a premature stop codon (20, 21). The catalytic component with cytidine deaminase activity is called Apolipoprotein B mRNA Editing Enzyme Catalytic Polypeptide 1 (APOBEC1). Although mRNA is the physiological substrate of this enzyme there is some evidence that it has activity on DNA *in vivo*. Misexpression of Apolipoprotein B mRNA Editing Enzyme in transgenic mice predisposes to cancer (22) and expression of human Apolipoprotein B mRNA Editing Enzyme in *E. coli* results in a mutator phenotype where there is a several 1000-fold enhanced mutation frequency seen at various loci in UNG-deficient strains. UNG is an enzyme involved in the repair of U:G mismatches caused by spontaneous cytosine deamination and deficiency in this enzyme prevents cells from repairing deaminated cytosines in their genome (23). Sequencing of DNA showed that mutations were triggered by conversion of cytosine to uracil in DNA. There appears to be some context specificity in the small stretches of DNA studied in this model (23) with a requirement for a 5'flanking pyrimidine. This is despite that fact that the cytosine base (#6666) exclusively targeted for deamination by this enzyme in the physiological RNA substrate has a 5'flanking purine (adenosine). Deamination of cytosines with 5'flanking pyrimidines by Apolipoprotein B mRNA Editing Enzyme may require factors not supplied in the *E. coli* model.

Recent work by Petersen-Mahrt & Neuberger (24) investigated the deamination activity of Apolipoprotein B mRNA Editing Enzyme *in vitro* on DNA substrates. They found no activity on double stranded DNA but cytosine bases in chemically synthesized single stranded DNA substrates were readily deaminated with 57% deamination of three cytosine bases in 120 minutes of incubation with a crude extract of enzyme. The activity of the enzyme appeared to be slightly higher when treated with RNase. The authors calculated that one molecule of Apolipoprotein B mRNA Editing Enzyme in their crude extract could deaminate a single cytosine base in a chemically synthesised single stranded DNA substrate in 10 minutes. They attributed this slow rate of deamination to the fact that their assay was likely to be sub-optimal. This was attributed to the lack of other factors required for activity that were not expressed in the *E. coli* host, that the human enzyme might not properly fold in the *E. coli* host, and the fact that any post-translation modifications required for activity would not be supplied by the *E. coli* host.

Activation-Induced Cytidine Deaminase (known as AID or AICDA) is a B-cell specific protein. Expression of Activation-Induced Cytidine Deaminase is a pre-requisite to class-switch recombination, a process mediating isotype switching of immunoglobulin, and somatic hypermutation, which involves the introduction of many point mutations into the immunoglobulin variable region genes. The mode of action of Activation-Induced Cytidine Deaminase is unknown. Current theories focus on the fact that Activation-Induced Cytidine Deaminase has sequence motif homology with Apolipoprotein B mRNA-Editing Enzyme and Cytidine Deaminase.

An early theory on the mode of action of Activation-Induced Cytidine Deaminase suggested that the hypothesised RNA-editing function of the enzyme might be involved in editing mRNAs that encode proteins essential for class-switch recombination and somatic hypermutation. The theory with most experimental support suggests that Activation-Induced Cytidine Deaminase functions as a DNA-specific cytidine deaminase. This model suggests that Activation-Induced Cytidine Deaminase deaminates cytosine bases in somatic hypermutation hotspot sequences to produce G:U mismatches and that these are differentially resolved to effect somatic hypermutation or class switch recombination (25). Evidence for the latter theory includes the suggestion that somatic hypermutation is initiated by a common type of DNA lesion, and that there is a first phase of hypermutation that is specifically targeted to dC/dG pairs. This would require Activation-Induced Cytidine Deaminase to have cytidine deaminase activity on DNA. All published work on Activation-Induced Cytidine Deaminase has focused on determining the *in vivo* substrate to elucidate the role of the enzyme in somatic hypermutation and isotype switching of immunoglobulin.

Research by various laboratories has showed that human Activation-Induced Cytidine Deaminase can deaminate cytosine on single stranded DNA *in vitro* (26-29) but not on single stranded RNA (26, 27). Activity of Activation-Induced Cytidine Deaminase on double-stranded DNA *in vitro* is limited to DNA coupled to transcription factors. It has been hypothesised that transcription allows deamination of double stranded DNA by generating secondary substrates that provide single-stranded DNA substrates such as stable R loops and stem loops (28). These secondary structures can be mimicked *in vitro* by producing bubbles, or loops, of centrally located noncomplementary regions of DNA, which will be single stranded, between complementary regions of double stranded DNA.

Activation-Induced Cytidine Deaminase deaminates cytosines in such bubbles. The efficiency of deamination depends on the length of the single stranded bubble. Bransteitter *et al.* (27) measured the percent of a chemically synthesised double stranded DNA substrate deaminated in 5 minutes of incubation and showed that substrates with 1 nucleotide bubbles were not deaminated, 3 nucleotide bubbles showed 5% deamination, 4 nucleotide bubbles showed 8 % deamination, 5 nucleotide bubbles showed 35 % deamination and 9 nucleotide bubbles showed 56 % deamination.

It has been hypothesised that Activation-Induced Cytidine Deaminase activity would be restricted to the physiological target (the immunoglobulin loci) because rampant DNA deaminase activity would be harmful to the cell. There is some suggestion that the deaminase activity of Activation-Induced Cytidine Deaminase is sequence specific (30), and it is hypothesised that Activation-Induced Cytidine Deaminase would show greatest activity on the somatic hypermutation hot-spot sequence RGYW (a sequence commonly mutated in the variable region of the immunoglobulin gene). Bransteitter *et al.* (27) showed that *in vitro* Activation-Induced Cytidine Deaminase had approximately three-fold higher activity on two hot-spot sequences compared with non-hot-spot sequences. Conversely, Dickerson *et al.* (26) found that the deaminase activity of Activation-Induced Cytidine Deaminase was sequence specific, but that cold-spot sequences (sequences of the variable region of the immunoglobulin gene that have never been found to be mutated *in vivo*) were deaminated equally well as hot-spot sequences, and that some hot-spot sequences were deaminated at only background levels.

Work by Pham *et al.* (31)tested the ability of Activation-Induced Cytidine Deaminase to deaminate cytosine bases *in vitro* using a large single stranded DNA template. In these experiments, the single stranded DNA template was a phage circular DNA substrate containing a 230-nucleotide target of the *lacZa* reporter sequence as part of a 365-nucleotide single-stranded gapped region. Incubations were carried out with 500 ng of the double-stranded phage DNA substrate with a 40-fold excess of enzyme in a 10 mM TRIS buffer (pH 8.0) with 1 mM EDTA and 1 mM dithiothreitol at 37°C for 20 mimutes. The spectra of mutations were assessed by transfecting mutated phage (which gave white or light blue plaques) into UNG-deficient *E. coli* with subsequent sequencing of clones. Under the test conditions used the deamination activity of Activation-Induced Cytidine Deaminase was found to vary with sequence context, and the authors hypothesised that

their results suggested the enzyme was a mobile molecule that processively deaminated cytosine molecules in the single stranded DNA.

Pham *et al.* (31) also described a protocol for measuring the deaminiation activity of Activation-Induced Cytidine Deaminase in a trancriptionally active version of their Phage substrate. Incubations were carried out with 30 nM of the double-stranded phage DNA substrate in a 50 mM HEPES buffer (pH 7.5) with 1 mM EDTA and 10 mM MgCl2 at 37°C for 30 minutes. The incubations included T7 RNA polymerase and rNTPs to produce transcriptionally active DNA which is a more accessible substrate for the Activation-Induced Cytidine Deaminase (27). These incubations showed that deamination mediated by Activation-Induced Cytidine Deaminase on the non-transcribed strand required RNA polymerase (active transcription) and that deamination on the transcribed strand, "protected" as an RNA-DNA hybrid, occurs at an approximately 15-fold lower rate. These incubations also demonstrated favoured deamination occurred in hotspot motifs.

Models that involve ectopic expression of Activation-Induced Cytidine Deaminase *in vivo* show untargeted cytosine deamination, that is deamination of genes other than the variable region of the immunoglobulin gene. For example, human Activation-Induced Cytidine Deaminase expressed in *E. coli*, which obviously lacks the human immunoglobulin target gene, produces context specific deaminations in genes screened for mutations (30). The reason for this context specific deamination was not examined, and it could be either a sequence specific requirement of Activation-Induced Cytidine Deaminase or it might be due to the secondary structure of different portions of the genomic DNA template (i.e. areas of single stranded DNA in actively transcribed genes would be deaminated with greater efficiency than stable double stranded DNA). It is possible that factors specifically expressed in B cells may be required for proper targeting of Activation-Induced Cytidine Deaminase mediated deamination to the immunoglobulin variable region.

Bransteitter *et al.* (27) recently incubated human Activation-Induced Cytidine Deaminase with a variety of chemically synthesized nucleic acid substrates *in vitro*. This work showed that, in a very simple model, Activation-Induced Cytidine Deaminase was capable of deaminating cytosine bases with 10-fold higher specific activity than 5-methylcytosine bases. The model involved incubating Activation-Induced Cytidine Deaminase with chemically synthesized single stranded DNA molecules with either 27 or

33 nucleotides, including either 1 or 2 cytosine bases, with no complimentary DNA strand present. These artificial substrates were present in high concentration, 100 nM, in a two-fold excess of Activation-Induced Cytidine Deaminase. The ability of Activation-Induced Cytidine Deaminase to differentially convert cytosine bases to uracil, with no or little activity on 5-methylcytosine, in a complex mixture of genomic DNA extracted from an individual where there are a multiplicity of mega-base fragments with a multiplicity of different sequence contexts of cytosine bases with both sense and complementary antisense strands present was neither tested nor considered.

The cytidine deaminase activity of Activation-Induced Cytidine Deaminase is inhibited by 1,10-phenanthroline, a strong chelator, but not by EDTA, a weaker chelator. This suggests that Activation-Induced Cytidine Deaminase requires a tightly bound metal ion, possibly zinc, for cytidine deaminase activity (27, 29). Activation-Induced Cytidine Deaminase retains cytidine deaminase activity over salt levels of 50 to 150 mM, can tolerate moderate levels of EDTA (5 to 10 mM), works at a wide range of pH (from 7.6 to 9.0 were tested) and works with varying efficiencies from room temperature to 37°C (26). These conditions are conducive to retaining the integrity of genomic DNA without fragmentation. Activation-Induced Cytidine Deaminase is still active after being heated at 65°C for 30 minutes (26).

Enzymes which modify DNA require only a few hours incubation. Purified restriction enzymes, for example, require only 1 hour incubation in optimal conditions to fully cleave double stranded DNA. Bransteitter *et al.* (27) measured 95 % conversion of cytosine to uracil by Activation-Induced Cytidine Deaminase in a chemically synthesized single-stranded DNA substrate in 16 minutes, and 56 % conversion of cytosine to uracil in a synthetic substrate with a 9 nucleotide single stranded bubble after 5 minutes. This is thus a fast reaction. Work by other groups, with different reaction conditions, have shown that only 10 % of a chemically synthesized single stranded DNA substrate containing one cytosine was converted to uracil after 30 minutes of incubation with Activation-Induced Cytidine Deaminase (26).

Summary of the Invention

In one aspect of the present invention there is provided a method for detecting the presence or level of alkylated cytosine in a sample of genomic or mitochondrial double stranded DNA from an individual, the method comprising:

(a) obtaining a sample of the double stranded DNA from the individual;

(b) converting at least one region of the double stranded DNA to single stranded DNA;

(c) reacting the single stranded DNA from step (b) with an enzyme, the enzyme differentially modifying alkylated cytosine and cytosine; and

(d) determining the level of enzymatic modification of the single stranded DNA.

Generally, the reaction conditions under which the enzyme is used will be such that the enzyme reacts substantially only with either alkylated cytosine or cytosine but not both. Preferably, the enzyme will be capable of reacting substantially with only one of alkylated cytosine or cytosine.

Preferably, the conversion of the at least one region of the double stranded DNA to single stranded DNA will comprise at least partially separating the two strands. The method may also comprise inhibiting annealing of the two strands of the double stranded DNA together once they have been separated, to facilitate access to the single stranded DNA by the enzyme.

One or more probes capable of hybridising with a respective strand of the double stranded DNA may be utilised to inhibit annealing of the separated strands. When a plurality of probes are used, the probes may hybridise with only one of the strands, or one or more of the probes may hybridise with one strand and the remaining probe or probes with the other strand.

Accordingly, a method of the invention may further comprise hybridising at least one probe with a strand of the double stranded DNA following separation of the two strands, to inhibit annealing of the strands together and thereby facilitate access to the single stranded DNA by the enzyme.

The probe will typically be an oligonucleotide. The probe may hybridise with a single contiguous region of a strand of the double stranded DNA, or separate discrete upstream and downstream regions of the strand which flank a target region of the strand being evaluated for the presence or level of alkylated cytosine.

In the former instance, at least two such probes will generally be utilised in a method of the invention, wherein one of the probes hybridises with a region of the strand downstream of the target region, and a further of the probes hybridises with a region of the strand upstream of the target region such that hybridisation of the other strand of the double stranded DNA to the target region is inhibited and the target region remains accessible to the enzyme.

In the latter instance, the probe will have a sequence such that when hybridised with the strand the spaced apart upstream and downstream regions of the strand are drawn toward each other or alternatively, the probe will have opposite end regions which hybridise with the strand and a middle region of non-complementary sequence that does not hybridise with the target region of the strand, such that a loop or bubble incorporating the target region is formed and hybridisation of the other strand of the double stranded DNA with the target region is thereby inhibited. To facilitate the formation of the loop or bubble, the middle region of the probe may incorporate inverted repeats that hybridise together following hybridisation of the probe with the strand.

To detect the presence or level of alkylated cytosine, the region or regions of the single stranded DNA reacted with the enzyme will typically be subjected to PCR and the resulting amplicon(s) analysed for sequence variations arising from the action of the enzyme. Hence, a method of the invention may further comprise:

amplifying at least one region of the single stranded DNA reacted with the enzyme, utilising a process involving thermocycling and primers to obtain an amplified product; and

analysing the amplified product for sequence modifications consistent with the presence of alkylated cytosine in the region or regions of the single stranded DNA amplified.

Determination of the level of alkylated cytosine may be achieved using any technique capable of detecting sequence modifications such as point mutations. Such techniques include, but are not limited to, nucleic acid sequencing and polymerase chain reaction (PCR) techniques, restriction enzyme digests, and techniques involving the use of probes that bind to specific nucleic acid sequences. The determination may comprise quantitative and/or qualitative analysis of the alkylated cytosine content of the target region of the single stranded DNA. In particular, hypermethylation or hypomethylation may be detected by a method of the invention and more particularly, patterns of cytosine alkylation in the DNA.

The DNA evaluated may comprise a gene or a region thereof and preferably, a regulatory non-coding region of a gene such as a promotor of a gene. Typically, the double stranded DNA will be genomic DNA.

Accordingly, in another aspect of the present invention there is provided a method for detecting the presence or level of alkylated cytosine in a sample of genomic DNA from an individual, the method comprising:

(a) obtaining a sample of genomic DNA from the individual;

(b) converting at least one region of the genomic DNA to single stranded DNA;

(c) reacting the single stranded DNA from step (b) with an enzyme, the enzyme differentially modifying alkylated cytosine and cytosine; and

(d) determining the level of enzymatic modification of the single stranded DNA.

In a still further aspect of the present invention there is provided a method for the diagnosis of a disease or condition in an individual involving detecting the presence or level of alkylated cytosine in a sample of genomic DNA from the individual, the method comprising:

(a) obtaining a sample of genomic DNA from the individual;

(b) converting at least one region of the genomic DNA to single stranded DNA;

(c) reacting the single stranded DNA from step (b) with an enzyme, the enzyme differentially modifying alkylated cytosine and cytosine; and

(d) determining the level of enzymatic modification of the single stranded DNA.

Typically, the enzyme used in a method of the invention will be a deaminase enzyme. The alkylated cytosine detected will generally be 5-alkylcytosine and usually, 5-methylcytosine. The presence of 5-methylcytosine is a useful marker in many conditions and disease states, and for upregulated or downregulated gene expression. Detection of the presence of 5-methylcytosine is also useful in mutation and epigenetic polymorphism analysis. Accordingly, the detection of 5-methylcytosine in DNA has significant diagnostic and other applications.

In yet another aspect there is provided a kit for use in a method of the invention, wherein the kit comprises one or more reagents for performing the method and instructions for use. The reagent or reagents may for instance be selected from the enzyme, buffers, primers for PCR and probes for separating the strands of the double stranded DNA utilised.

The term "individual" as used herein is to be taken in the broadest sense and is intended to include within its scope human beings and non-human animals, bacteria, yeast, fungi and viruses.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer, or step, or group of elements, integers or steps.

The features and advantages of methods falling within the scope of the present invention will become further apparent from the following description of preferred embodiments of the invention.

Detailed Description of the Invention

Generally, the enzyme used in a method at the present invention will have cytidine or cytosine deaminase activity, and be able to deaminate cytosine bases in genomic DNA to uracil without substantially deaminating any 5-methylcytosine bases in the DNA. The enzyme may be a thermostable cytidine or cytosine deaminase derived from a thermophilic organism.

The enzyme may for instance be selected from Activation-Induced Cytidine Deaminase, Cytidine Deaminase (also known as Cytidine Aminohydrolase EC 3.5.4.5), Cytosine Deaminase (also known as Cytosine Aminohydrolase EC 3.5.4.1), Deoxycytidine Deaminase (also known as Deoxycytidine Aminohydrolase EC 3.5.4.14), Deoxycytidilate Deaminase (also known as Deoxycytidilate Aminohydrolase), Apolipoprotein B DNA Editing Enzyme (ApoBRe) and catalytic fragments, homologues and variants thereof. By catalytic fragment is meant an enzyme fragment possessing some or all of the catalytic activity of the complete enzyme. Generally, a catalytic fragment utilised in a method of the invention will have substantially the same catalytic activity as the complete enzyme. Catalytic fragments of ApoBRe include APOBEC1. Homologues of APOBEC1 include APOBEC2 and APOBEC3A to APOBEC3G, and one or more of such homologues may also be utilised in a method described herein.

Genomic DNA will usually be utilised in a method of the invention and may be extracted from any cells or biological samples deemed appropriate. Genomic DNA extracted by standard protocols is fragmented to varying degrees and is largely double stranded. Activation-Induced Cytidine Deaminase, and other enzymes with cytidine deaminase activity, typically have highest activity on single stranded DNA, or on regions of single stranded loops in double stranded DNA (27). Double stranded DNA can be made single-stranded by a variety of methods including heat denaturation, chemical denaturation, protein binding and exonuclease activity and any of these techniques may be utilised.

Heat denaturation is commonly used for generating single-stranded DNA and is used in processes such as PCR. Chemical denaturation involves incubations in chemicals such as alkali (7, 32) or formamide (32). Incubation with proteins that bind single-stranded DNA such as such as Bacteriopharge T4 gene 32 protein (and truncated forms of this protein) destabilise the double helix of genomic DNA and reduces secondary structures (33, 34). Enzymatic denaturation involves selective enzymatic degradation of one strand of double stranded DNA by incubation with exonucleases such as exonuclease III from *E. coli* which catalyses the 3'to 5' removal of mononucleotides from 3'-hydroxy termini of duplex DNA. Exonulease III has been used to prepare single-stranded DNA substrates for dideoxy sequencing (35), direct sequencing using MALDI-TOF mass spectroscopy (36) and single-strand conformation polymorphism analysis (32).

Probes utilised for hybridising with the single standed DNA generated by separation of the strands of the genomic DNA for inhibiting the annealing of the separated strands and thereby allowing access of the enzyme to the target region of interest, will generally be synthetic oligonucleotide probes. The probes may be DNA probes or analogues thereof such as RNA, PNA, or LNA probes or chimeras thereof. Typically the probes will be incapable of acting as primers and being extended during PCR. The probes will generally be about 10 bases in length, usually between about 10 and 50 bases in length and preferably, be about 17 to about 30 bases in length. However, longer probes are not excluded and may be used for generating a plurality of bubbles along the length of the DNA strand to be assayed for facilitaing reaction of the enzyme with multiple sites along the strand.

Incubation of the single stranded genomic DNA with an enzyme with a preferential ability to deaminate cytosine and not 5-methylcytosine results in a sequence with uracil in place of cytosine residues but with 5-methylcytosine residues substantially unchanged. The optimum reaction conditions for reaction of the DNA with the selected deaminase enzyme may be determined by altering one or more reaction conditions utilised.

Previously published work with Activation-Induced Cytidine Deaminase and the APOBEC1 catalytic polypeptide and its homologues has shown no attempt to optimise the reaction conditions under which the enzyme and its substrates are incubated. Work in *in vivo* models, such as *E. coli* transformants, allow investigators little control over reaction conditions. Genomic DNA from the colorectal cancer cell line SW480 shows complete 5-methylation of the cytosines present in CpG sites in the CpG island in the promoter region of the *p16* gene. The DNA in this region of the gene also contains unmethylated cytosines. Genomic DNA from this cell line therefore provides a model substrate on which to test reaction variables to determine the optimum conditions for maximum discrimination between cytosine and 5-methylcytosine incorporated into DNA as substrates for deamination by enzymes with cytidine deaminase activity.

More particularly, for deteremining optimum reaction conditions, genomic DNA is first extracted from the cells using standard methods. The DNA from SW480 cells is then converted to single stranded DNA, preferably by heat denaturation. The re-annealing of the separated strands can be inhibited using probes as described above. The enzyme to be

tested is subsequently added to the single stranded DNA and incubated under a range of variables selected from for instance; the concentration of DNA, the concentration of enzyme, the time of incubation, the temperature of incubation, the composition and concentration of the buffering ion (commonly used buffers include TRIS, HEPES, MOPS & imidazole), the pH of the buffer (from pH 4.0 to 10.0), the concentration and type of salt (commonly used salts include sodium chloride, sodium acetate, potassium chloride, potassium acetate, salts of sulphate and salts of ammonium), the concentration of various cationic metal ions (for example magnesium, manganese, lead, and calcium), the concentration of various protein stabilisers if any (for example reducing agents such as dithiothreitol (DTT), other proteins (such as bovine serum albumin (BSA)), sugars (such as sucrose, maltose, glucose, trehalose, glycerol and fructose), detergents (such as Triton®X-100 and Tween-20), and co-solvents (such as proline, betaine, formamide, DMSO, alcohols and polyols). The degree of discrimination between cytosine and 5-methylcytosine achieved using different combinations of these variables can then be assessed by protocols as further described below. Those skilled in the art will appreciate that the above list of reaction condition variables is not exclusive and further examples of reagents and conditions that alter or enhance substrate specificity and rates of reaction of enzymes can be found in publicly available literature.

Following incubation of the test DNA with the enzyme, the target region of interest in the DNA will typically be amplified by PCR. Generally, the enzyme will be heat denatured before the commencement of the PCR. Using the modified DNA as a template in PCR results in an amplified sequence (amplicon) with thymidine residues in place of cytosine in the original sequence and cytosine in place of 5-methylcytosine. Accordingly, the conversion of cytosine bases to uracil by the enzyme, followed by conversion to thymine by the PCR, creates a modified DNA with sequence differences associated with the methylation status of the cytosines in the original DNA template.

These sequence variations can be detected using any protocol which can discriminate between thymidine and cytosine bases, including techniques such as direct sequencing of the region (e.g. see Herman *et al* (10)), digestion of the PCR amplicon with restriction enzymes, methylation-specific PCR (10), Restriction Endonuclease Mediated Selective PCR (REMS-PCR) (eg.(37); International Patent Application No. PCT/AU96/00213) and hybridisation with methylation-specific probes (11). Methylation-

specific PCR relies on primers that take advantage of the sequence differences between methylated and non-methylated regions after conversion by the enzyme. All of these methods will give information on the methylation status of cytosines in the target region of the test DNA being assayed.

The selective nature of amplification by REMS-PCR means that it is well suited for analysis of rare genetic variations such as tumour sequences in a background of normal sequences, or foetal sequences in a background of maternal sequences. Accordingly, a method of the invention may form the basis of minimally invasive assays in which body fluids are analysed for the presence of variant sequences characterised by altered or aberrant cytosine methylation patterns.

The method of the present invention may be used to detect hypermethylated sequences within the promoter region of genes in association with human tumours such as for example, hypermethylation in the CpG island within the *p16* gene promoter. Hypermethylation of this region has been detected in bladder, breast, gastric, head & neck, oesophageal, colon, lung and liver cancer. Other examples of genes which have CpG island hypermethylation in association with human tumours include *E-cadherin* (breast, prostate, colon, bladder, and liver tumours), the von Hippel Lindau (VHL) gene (renal cell tumours), *BRCA1* (breast tumours), p15 (leukemias, Burkitt lymphomas), hMLH1 (colon tumours), ER (breast, colon, lung tumours, and leukemias), HIC1 (brain, breast, colon, and renal tumours), MDG1 (breast tumours), GST-π (prostate tumours), O^6-MGMT (brain tumours), calcitonin (carcinoma and leukemia), and *myo-D* (bladder tumours) (1, 3).

A method as described herein can also be used to identify regions of hypomethylation, such as regions of hypomethylation associated with the transcriptional activation of genes such as urokinase or S100A4 in cancer.

Accordingly, altered methylation patterns may be used as markers of tumour cells. Specific applications utilising such markers include for example, minimally invasive screening or early diagnosis of tumours or cancers, detection of micrometastatic or metastatic disease in lymph nodes, detection of unresected tumour cells at tumour margins or other residual disease, or as a tool for predicting relapse. In addition, differences in patterns of 5-methylcytosine bases at discreet genetic loci may be used as a marker for foetal DNA or disease states such as fragile X syndrome and altered gene imprinting states.

The presence of 5-methylcytosine may also provide a marker of endogenous or exogenous DNA associated with viruses, bacteria or other pathogens and so provide a means indicating infection by the pathogen or of identifying the pathogen.

Control DNA sequence of known cytosine methylation status will generally be utilised for assessing the efficacy of the enzymatic modification of cytosine bases. Suitable controls include plasmids, PCR fragments generated by replacing dCTP with methy5-dCTP (38), and commercially available human genomic that is DNA universally methylated for all genes (CpGenome™ Universally Methylated DNA, Intergen Company, Cat. No. S7821). In addition, cell line DNA, extracted from cell lines with a known methylation status may be used for positive and negative controls. As an example, the CpG dinucleotides in the CpG island in the promoter region of the *p16* gene are fully methylated in the lung cancer cell lines H157 and U1752, and unmethylated in the lung cancer cell lines H249 and H209 (10). The genomic DNA maybe extracted from the cell lines by standard protocols known in the art.

Enzymatic modification of cytosine bases in the test DNA being assayed will generally be carried out using the minimum incubation period deemed necessary to achieve modification of the cytosine bases in the DNA by the enzyme utilised, and in conditions that do not lead to excessive fragmentation of the DNA. Advantageously, the protocol will typically be faster than conventional DNA modification protocols known in the art.

The disclosures of all references referred to are specifically incorporated herein by cross reference. The present invention will now be further described with reference to the following non-limiting examples.

Example 1: Enzymatic conversion of genomic DNA using Activation-Induced Cytidine Deaminase for the detection of the methylation status of the CpG Island in the promoter of the *p16* (INK4a) gene.

Genomic DNA is first extracted from a blood or tissue sample from the individual using a standard extraction protocol known in the art. Human genomic DNA, universally methylated for all genes (CpGenome™ Universally Methylated DNA), is used as a positive control for detection of 5-methylcytosine within the CpG island in the promoter of the *p16* gene.

Single stranded DNA is generated from the double stranded genomic DNA by heat denaturation. The resulting single-stranded DNA is subsequently incubated with Activation-Induced Cytidine Deaminase in conditions that promote deamination of cytosine bases in the DNA, but not 5-methylcytosine bases. Activation-Induced Cytidine Deaminase can be prepared in a number of ways including as a crude extract from activated B-cells (28), and expression of a fusion protein to facilitate purification (26, 27).

The area of interest around the CpG island of the *p16* promoter (GenBank Accession No. X94154) is then amplified by PCR. Primers are chosen in regions that are not methylation hot-spots to reduce the possibility of efficiency of amplification being dependent on methylation status. Suitable primer sequences are described in Herman *et al.* (10). The PCR product contains thymidine bases where unmethylated cytosine existed in the template genomic DNA and cytosine bases where 5-methylcytosine bases existed in the template genomic DNA. The methylation status of the CpG island in the promoter region of the *p16* gene is then assessed using a suitable protocol as described above. Detection of methylated CpG sequences within the CpG island in the promoter region of *p16* may used as a marker of tumours of several organs including the bladder, breast, gastric, head & neck, oesophageal, colon, lung or liver.

Example 2: Enzymatic conversion of genomic DNA using Activation-Induced Cytidine Deaminase to facilitate detection of the methylation status of the individual CpG dinucleotides in the CpG island in the promoter of the *p16* (INK4a) gene.

As in Example 1, genomic DNA is first extracted from a blood or tissue sample from the individual using a standard extraction protocol known in the art. Human genomic DNA, universally methylated for all genes (CpGenome™ Universally Methylated DNA), is used as a positive control for detection of 5-methylcytosine within the CpG island in the promoter of the *p16* gene (also called the CDKN2 gene, GenBank Accession No. X94154).

Specific areas of the CpG island in the promoter of the *p16* gene are targeted for enzymatic conversion by Activation Induced Cytidine Deaminase by using a synthetic DNA probe with areas of complementarity around the CpG sequence to be analysed such that hybridization of the DNA probe produces a central loop of single stranded DNA containing the CpG sequence, or sequences, to be analysed. The DNA probe is hybridized

to the genomic DNA by mixing the probe and the genomic DNA together, then heat denaturing the genomic DNA and cooling the solution to a temperature lower than the melting-temperature of the probe. In a variation of this technique, a plurality of such DNA probes may be hybridised with the genomic DNA to target a number of regions of interest in the genomic DNA. In a further variation of this technique, the probes may contain modified DNA bases such as PNA or LNA.

The genomic DNA with the DNA probe hybridised to it is subsequently incubated with Activation-Induced Cytidine Deaminase under conditions that promote deamination of cytosine bases in the genomic DNA by the enzyme, but not 5-methylcytosine bases.

The area of interest around the CpG island of the *p16* promoter is then amplified by PCR. The PCR product will contain thymidine bases where unmethylated cytosine existed in the loop of template genomic DNA, and cytosine bases where 5-methylcytosine bases existed in the template genomic DNA. The methylation status of the CpG island in the promoter region of *p16* is then assessed as in Example 1.

Methylation-specific PCR relies on primers that take advantage of the sequence differences between methylated and unmethylated regions after conversion by an agent such as bisulfite. To detect the CpG dinucleotides targeted for enzymatic conversion by Activation Induced Cytidine Deaminase using methylation specific PCR, methylation-specific primers are designed to this region.

Although the present invention has been described hereinbefore with reference to a number of preferred embodiments, the skilled addressee will appreciate that numerous changes and modifications are possible without departing from the spirit or scope of the invention. The present embodiments described are, therefore, to be considered in all respects as illustrative and not restrictive.

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Claims

1. A method for detecting the presence or level of alkylated cytosine in a sample of genomic or mitochondrial double stranded DNA from an individual, the method comprising:

(a) obtaining a sample of the double stranded DNA from the individual;

(b) converting at least one region of the double stranded DNA to single stranded DNA;

(c) reacting the single stranded DNA from step (b) with an enzyme, the enzyme differentially modifying alkylated cytosine and cytosine; and

(d) determining the level of enzymatic modification of the single stranded DNA.

2. A method according to claim 1, wherein the double stranded DNA is genomic DNA.

3. A method according to claim 1 or 2 wherein the single stranded DNA is reacted with the enzyme under conditions such that the enzyme reacts substantially only with either alkylated cytosine or cytosine in the single stranded DNA but not both.

4. A method according to claim 1 or 2 wherein the enzyme is capable of reacting substantially with only one of alkylated cytosine or cytosine in the single stranded DNA.

5. A method according to any one of claims 1 to 4 wherein the conversion of the at least one region of the double stranded DNA to the single stranded DNA comprises at least partially separating the two strands of the double stranded DNA.

6. A method according to claim 5 further comprising inhibiting annealing of the two strands of the double stranded DNA together once they have been separated, to facilitate access to the single stranded DNA by the enzyme.

7. A method according to claim 6 further comprising hybridising at least one probe with a strand of the double stranded DNA following separation of the two strands, to thereby inhibit the annealing of the two strands together and facilitate access to the single stranded DNA by the enzyme.

8. A method according to claim 7 wherein at least two said probes are hybridised with the strand of the double stranded DNA, one of the probes hybridising with a region of the strand downstream of a target region being evaluated for the presence or level of alkylated cytosine, and a further of the probes hybridising with a region of the strand upstream of the target region such that hybridisation of the other strand of the double stranded DNA to the target region is inhibited and the target region remains accessible to the enzyme.

9. A method according to claim 7 wherein the probe hybridses with upstream and downstream regions of the strand which flank a target region of the strand being evaluated for the presence or level of alkylated cytosine, and the hybridisation of the probe with the strand draws the upstream and downstream regions of the strand toward each other such that a loop or bubble incorporating the target region is formed.

10. A method according to claim 6 wherein the probe hybridises with the strand of the double stranded DNA either side of a target region of the strand being evaluated for the presence or level of alkylated cytosine and the probe has a middle region of non-complementary sequence that does not hybridise with the target region of the strand, such that a loop or bubble incorporating the target region is formed.

11. A method according to claim 10 wherein the middle region of the probe incorporates inverted repeats that hybridise together following hybridisation of the probe with the strand of the double stranded DNA.

12. A method according to any one of claims 1 to 6 wherein the determination of the level of enzymatic modification of the single stranded DNA comprises subjecting at least one region of the single stranded DNA reacted with the enzyme to an amplification process involving thermocycling and primers to obtain an amplified product, and analysing the amplified product for sequence variations arising from the enzymatic modification of the single stranded DNA.

13. A method according to claim 12 wherein the analysis of the amplified product comprises subjecting the amplified product to a technique selected from the group consisting of nucleic acid sequencing, polymerase chain reaction techniques, restriction enzyme digests and techniques involving the use of probes that bind to specific nucleic acid sequences.

14. A method according to claim 13 wherein the analysis of the amplified product comprises subjecting the amplified product to a polymerase chain reaction technique.

15. A method according to any one of claims 1 to 14 comprising detecting the presence or level of alkylated cytosine in a gene or a non-coding region of a gene, or a fragment thereof.

16. A method according to claim 15 comprising detecting the presence or level of alkylated cytosine in a promotor for regulating expression of a gene.

17. A method according to any one of the claims 1 to 16 further comprising diagnosing a disease or condition in the individual on the basis of the presence or the level of the alkylated cytosine in the sample of genomic double stranded DNA.

18. A method according to any one of claims 1 to 17 wherein the enzyme is a deaminase enzyme.

19. A method according to any one of claims 1 to 18 wherein the alkylated cytosine is methylated cytosine.

20. A method according to claim 19 wherein the methylated cytosine is 5 methylcytosine.

Dated this 4th day of July 2003

Johnson & Johnson Research Pty Limited
By their Patent Attorneys
Blake Dawson Waldron Patent Services

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